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| 14. ABSTRACT<br>The primary objective of our <i>Early Investigator Synergistic Idea Award</i> is to establish a driver prioritization pipeline to functionally evaluate lung cancer genomics data to identify somatic driver aberrations, which beyond the handful of well-characterized genes like oncogenic <i>KRAS</i> , contribute to lung cancer progression. As outlined in our Annual Report, we have successfully constructed the necessary gene libraries for the screens outlined in our proposal, optimized and completed the proposed <i>in vitro</i> cell invasion screens and will complete full analysis on the <i>in vivo</i> metastasis screens by the time of our Final Report. These combined efforts have revealed several robust drivers of cell invasion and <i>in vivo</i> metastasis that will be 1) developed into hypothesis-driven projects funded by additional award mechanisms and 2) fuel target biology studies aimed at translating our findings into new patient therapies. We have also made a number of key technical advanced over the course of this project that will have a sustained impact on screening of lung cancer genomics data beyond the life of this particular award. |                  |                         |                                      |                                          |                                            |
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## **INTRODUCTION**

Cancer cells are endowed with diverse biological capabilities driven by an ensemble of inherited, somatic and epigenetic aberrations. As we enter the era of personalized medicine, characterization of the cancer genome has begun and will continue to influence diagnostic and therapeutic decisions in the clinic. Genome profiling technologies are generating a compendium of genomic aberrations in major cancer lineages with the goal of identifying the most promising therapeutic targets and diagnostic biomarkers. The game changing output from these technologies is radically transforming cancer science. At the same time, these efforts are revealing the complexity of cancer genomes, which are comprised of causal “driver” aberrations and many more biologically neutral “passengers” that arise through the unstable nature of tumor genomes. While most cancers acquire one or more well-studied, high frequency driver events (e.g., mutations/gene copy number changes in *KRAS*, *TP53*, *EGFR*, *MYC*, *BRAF*, etc.), much less is known about the overly abundant low frequency (<5%) aberrations contributing to tumor progression and response to therapeutics. Comprehensive biological assessment of low frequency aberrations is difficult given their large number and the fact that they may either directly drive tumor progression or indirectly influence tumor behavior through modifying activities of other drivers like *KRAS*. Moreover, distinguishing driver events from passengers is further complicated by the fact that driver events are shaped by the specific biological context of a given cancer, including its tissue type, microenvironment and other host determinants including the immune system. The primary objective of our *Early Investigator Synergistic Idea Award* is to establish a **driver prioritization pipeline** to functionally evaluate lung cancer genomics data to identify somatic driver aberrations, which beyond the handful of well-characterized genes like oncogenic *KRAS* and *EGFR*, likely contribute to lung cancer progression, invasion, and metastasis. Our study integrates genetically engineered mouse models of lung cancer, genomics data generated by The Cancer Genome Atlas (TCGA) and functional screens to identify drivers of lung cancer progression. We are using these tools to implement a novel, scalable screening infrastructure that permits high-content, gain-of-function screens to accelerate validation of functional somatic aberrations. This work is possible through advances made in our laboratory that include (1) high-throughput, highly accurate modeling of somatic aberrations into our collection of over 32,000 sequence-verified human genes and (2) a novel molecular barcoding approach that facilitates cost-effective detection of driver events following *in vitro* and *in vivo* functional screens. Our Specific Aims are as follows: (1) Construction of a lung cancer somatic driver library; (2) Functional screens for drivers of lung cancer metastasis; (3) Clinicopathological prioritization and validation of top candidates.

**Herein we describe our progress over the final 12 months of this two-year project. Importantly, because of the collaborative nature of this “Synergy” project involving two principal investigators (Scott and Gibbons), this Annual Report covers work primarily completed in the Scott laboratory. Work conducted in the Gibbons laboratory will be included in a separate Annual Report where indicated.**

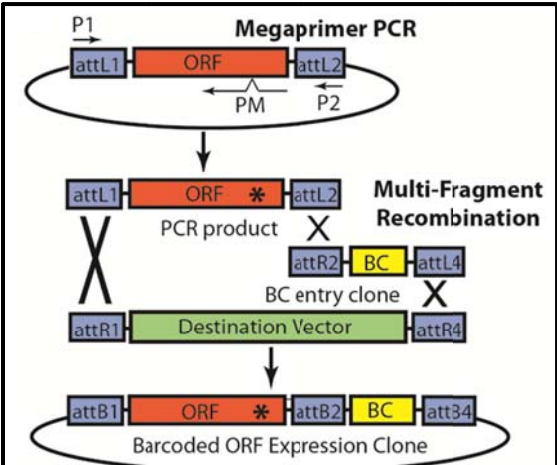
## **BODY**

**Subaim 2.1 *In vitro* screens for cell invasion and anoikis resistance (proposed completion, months 6-12) – strategically delayed from year 1 to year 2; progress reported here.**

As described in our first Annual Report, we completed Aim 1 by employing our High-Throughput Mutagenesis and Molecular Barcoding (HiTMMoB) technology (Fig. 1) to successfully generate an arrayed open reading frame (ORF) library consisting of 279 sequence-verified wild-type and mutant ORF donor clones based on computational mining of murine and human lung cancer genomics data. These ORFs were subsequently recombined into the pLentiEF6.3-puro lentiviral vector specially constructed for this project. This vector is compatible with our flexible molecular barcoding technology (Fig. 1), thus each ORF was uniquely tagged with a 24-nucleotide DNA “barcode” followed by high-

throughput DNA sequencing from bacteria to confirm each barcode and the identity of its associated ORF. Validated barcoded ORFs were re-arrayed into a 96-well format compatible with lentivirus production for the proposed studies.

In this Subaim we proposed to perform parallel *in vitro* screens for gene drivers of cell invasion and anoikis resistance using cells transduced with individual ORFs constructed in Aim 1. As described in our first Annual Report, we fully optimized the invasion screening assay and had initiated ORF screening. We initially proposed to complete the *in vitro* assays during the first year; however, we determined that screening would be more efficient if conducted in a “rolling” fashion whereby individual candidate pools (e.g., 20-30 gene sets of the 279 library panel) were entered into both the *in vitro* and *in vivo* screens simultaneously, thus eliminating the costly need to construct the transduced cell line panel on two separate occasions. Therefore, while strategically delayed based on the Statement of Work, we report here completion of the invasion screen and discontinuation of the anoikis screen (discontinuation also discussed in our first Annual Report).



**Figure. 1. HiTMMoB (High-Throughput Mutagenesis and Molecular Barcoding).** Schematic illustrating our scalable mutagenesis and barcoding approach applicable to our collection of ~32,000 ORFs. BC = Barcode; att = recombination sequences for cloning; P1, PM, P2 = mutagenesis DNA primers

Cell invasion Careful optimization assays (see first Annual Report) led us to infect 393P cells with individual lentivirus carrying a total of 224 genes for selection in puromycin for stable expression. We were unable to obtain stable cell lines for 55 of the 279 ORFs given virus titer deficiencies associated with large ORF size or cell toxicity upon expression (data not shown). Successful stable lines were cryo-banked for (1) future use in functional and biochemical validation assays and (2) *in vivo* screening assays proposed in Subaim 2.2. For invasion assays, 50,000 cells were seeded in quadruplicate across 96-well cell invasion plates containing Matrigel to simulate extracellular matrix (BD Bioscience). Plates were incubated at 37°C for 28 hrs, and the degree of cell invasion promoted by each ORF compared to negative controls cells (mCherry-expressing cells; seeded into eight replicate wells) was determined by measuring the fluorescence of total invaded cells on the bottom of each chamber using a compatible plate reader. Primary screen hits were statistically analyzed by comparing the standard deviation across all plates of all negative control wells (mCherry) and using a threshold of 0.5 standard deviations above mCherry normalized back to individual plates accounting for plate to plate variations with a significant p-value of 0.01. This provided a list 40 ORFs that scored at this threshold revealing 39 potent invasion drivers (Table 1) currently under secondary validation (see Subaim 3.1). Among top invasion drivers identified in the primary screen are *bona fide* oncogenes (e.g., *KRAS*, *MYC*),

| Rank | Gene ID  | Fold Invasion | Rank | Gene ID | Fold Invasion |
|------|----------|---------------|------|---------|---------------|
| 1    | IKBKB    | 2.6           | 21   | SEPP1   | 6.7           |
| 2    | PHC3     | 3.3           | 22   | ZNF281  | 7.0           |
| 3    | ENY2     | 3.8           | 23   | RNF115  | 7.4           |
| 4    | KDELR2   | 3.8           | 24   | ADIPOR1 | 8.3           |
| 5    | GGCT     | 3.9           | 25   | FMOD    | 8.6           |
| 6    | KRAS     | 3.9           | 26   | YWHAZ   | 9.3           |
| 7    | PRKACB   | 4.0           | 27   | JRK     | 9.7           |
| 8    | SLA      | 4.3           | 28   | SRP54   | 10.1          |
| 9    | MBIP     | 4.3           | 29   | GCK     | 10.3          |
| 10   | DTL      | 4.7           | 30   | MAPK6v2 | 11.2          |
| 11   | SFRP4    | 4.7           | 31   | SETDB1  | 11.6          |
| 12   | VAMP4    | 4.8           | 32   | PTK2    | 11.8          |
| 13   | TMEM106B | 4.9           | 33   | FCGR2B  | 12.8          |
| 14   | KLHL7    | 5.2           | 34   | CABLES1 | 16.2          |
| 15   | MAPK6v1  | 5.4           | 35   | CCNE1   | 17.0          |
| 16   | FOXO3    | 5.5           | 36   | CBLB    | 18.3          |
| 17   | TRAPPC6B | 5.6           | 37   | ATP1A2  | 23.7          |
| 18   | S100A1   | 5.6           | 38   | DAB2    | 30.7          |
| 19   | MYC      | 5.8           | 39   | IMPAD1  | 32.6          |
| 20   | DFNA5    | 6.4           | 40   | SNAI2   | 37.4          |

**Table 1. Summary of *in vitro* invasion screen top hits.** See text for details.

Among top invasion drivers identified in the primary screen are *bona fide* oncogenes (e.g., *KRAS*, *MYC*),

metastasis genes (e.g., *SNAI2*) and others associated with cancer progression. Notably, the majority of genes identified by our invasion screen have not been directly associated with cancer metastasis and will therefore be subjected to hypothesis-driven research fueled by future funding mechanisms. A number of these genes are predicted druggable (e.g., *MAPK6*) and will therefore be immediately assessed in target biology assays. **We will provide additional discussion on predicted mode-of action for candidates passing secondary validation in our Final Report.**

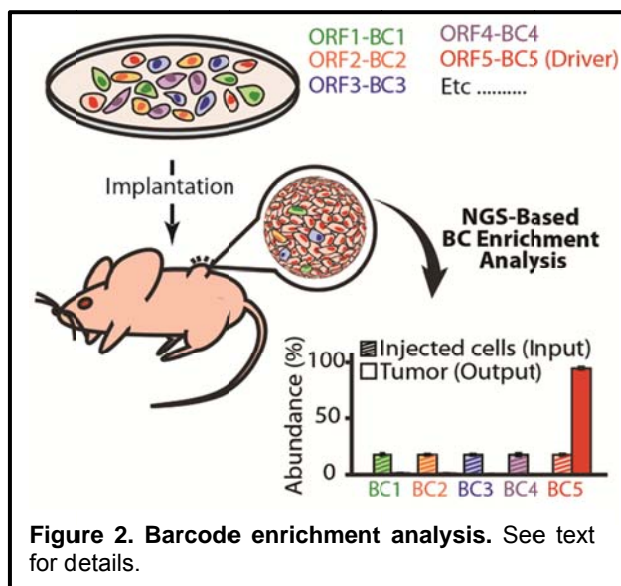
**Anoikis Resistance:** As described in our first Annual Report, we made great efforts to develop the proposed anoikis-resistance assays but had been unsuccessful due to the fact that all cell lines assayed (e.g., 393P, 393LN, etc.) already exhibited robust anoikis resistance and were therefore not suitable as screening models. During Year 2 we examined our newly-derived HBEC cell model (see first Annual Report) to assess its performance in this assay. Unfortunately, this line also exhibited high baseline anoikis resistance rendering it unsuitable for screening (data not shown). In our last report we described an alternative 3D culture assay, which would be used in place of the anoikis assay if problems persisted, whereby cells are grown on a bed of extracellular matrix. The Gibbons laboratory has previously published that this method better mimics the *in vivo* condition by modeling the cell-matrix interactions, is scorable for cell growth and invasion, and can be modified by adjusting the composition and biophysical properties of the matrix [1-3]. **The Gibbons laboratory has continued these 3D assays to validate ORFs scoring in our invasion assays and will present those findings in their Annual and Final Reports.**

### Subaim 2.2. *In vivo* positive selection screens (proposed completion months 3-15).

In this Subaim we propose to perform *in vivo* metastasis screens with pooled viral-infected 393P cells to positively select for single and combinatorial drivers of metastasis. In year 1 we completed pilot assays to determine the optimal *in vivo* screening conditions (see first Annual Report). Based on pilot experiments of optimal tumor cell inoculum, each group of injections included 10 mice that were injected subcutaneously with a pool of  $10^6$  cells each, comprised of ORF expressing cell lines (30-50,000 cells/ORF) plus one additional mCherry control cell line used as an internal control. With each experimental pool we also injected 5 additional mice as negative control with  $10^6$  cells expressing mCherry only. Mice were observed for general health and the growth of primary subcutaneous tumors was recorded. The animals were sacrificed once tumors reached maximum tumor burden or upon signs of deterioration in general health as specified in our IACUC-approved animal protocol.

We have now entered all 224 stable ORF cell lines (see above) across 12 pools into the *in vivo* screening assay (n=120 pool-injected mice). All mice developed subcutaneous tumors as expected for 393P cells, and negative control mice never developed distal lung metastases similar to our pilot assays. Of the 105 pool-injected mice euthanized thus far, we observed 58 mice with varying numbers of lung macrometastases.

Metastasis drivers enriched in metastases are identified by barcode (BC) sequencing. Briefly, each ORF-associated BC serves as a surrogate identifier for its associated ORF thus permitting individual ORF detection by next generation sequencing (NGS) using an Ion Torrent Personal Genome Machine (PGM). Individual ORF enrichment (positive selection) among pooled ORFs can be determined by comparing the number of individual BC reads between populations as a ratio of each BC to the total number of total BC reads per sequenced amplicon. For example, in the case of *in vivo* tumor driver screens (Fig. 2) as used here, ORF-driven tumors/metastases (output) will be positively enriched for driver-associated



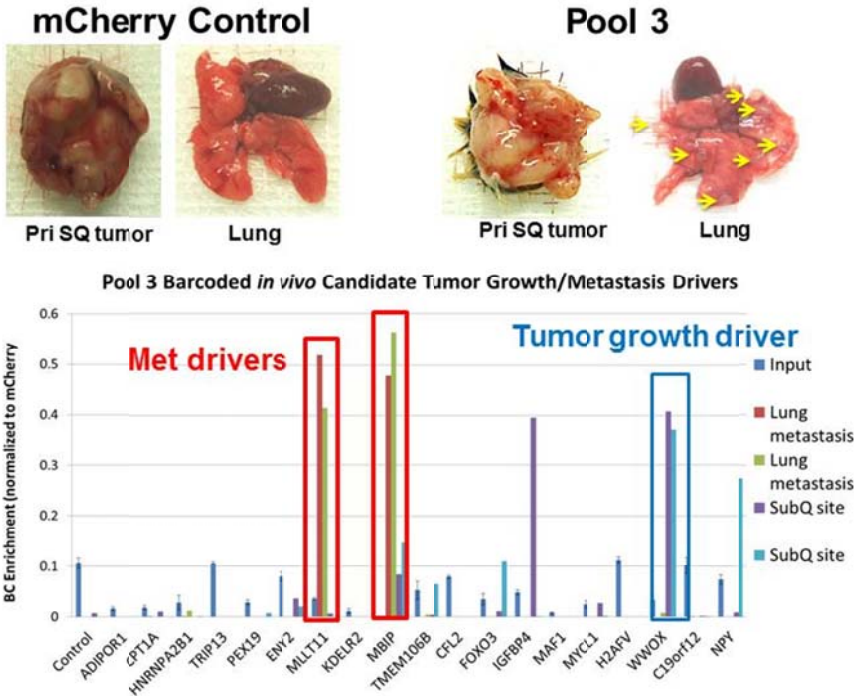
**Figure 2. Barcode enrichment analysis.** See text for details.

BCs (BC5) and lose those with no role in progression (i.e., passengers, BC1-4) compared to injected cells (input) carrying all barcoded ORFs.

We have processed input and output samples for 11 of 12 pools (58 metastasis-bearing mice) and have completed BC enrichment analysis using input (injected cells), output (2 cores each of primary tumor and metastatic lungs) for pools 3 and 4. Analysis of pools 3 and 4 identified a total of 8 metastasis drivers enriched in anywhere for 1 to 7 mice in each representative pooled cohort (Table 2). Shown in Figure 3 is a representative example: Mouse #8 injected with ORF pool 3. BC analysis revealed equal and robust enrichment for two metastasis drivers, *MLLT11* and *MBIP*, in two separate lung tissue cores. *MLLT11* and *MBIP* were not enriched in either primary tumor core analyzed, indicating that these specifically enhanced distal metastasis. In contrast, we also discovered enrichment of *WWOX* in primary tumors, suggesting that expression of this ORF promoted tumor growth activity but not distal metastasis.

| Pool         | Clones Injected   | Mice Injected | Mice Euthanized | Mice alive with tumors | Mice with Metastases | Identified metastasis drivers (by sequencing) |
|--------------|-------------------|---------------|-----------------|------------------------|----------------------|-----------------------------------------------|
| 1            | 19+Mcherry        | 10            | 10              | 0                      | 2                    | TBD                                           |
| 2            | 19+Mcherry        | 10            | 10              | 0                      | 2                    | TBD                                           |
| 3            | 19+Mcherry        | 10            | 10              | 0                      | 8                    | 6                                             |
| 4            | 19+Mcherry        | 10            | 10              | 0                      | 8                    | 2                                             |
| 5            | 19+Mcherry        | 10            | 10              | 0                      | 3                    | TBD                                           |
| 6            | 19+Mcherry        | 10            | 10              | 0                      | 2                    | TBD                                           |
| 7            | 19+Mcherry        | 10            | 10              | 0                      | 9                    | TBD                                           |
| 8            | 19+Mcherry        | 10            | 7               | 3                      | 4                    | TBD                                           |
| 9            | 19+Mcherry        | 10            | 9               | 1                      | 7                    | TBD                                           |
| 10           | 19+Mcherry        | 10            | 10              | 0                      | 6                    | TBD                                           |
| 11           | 19+Mcherry        | 10            | 9               | 1                      | 7                    | TBD                                           |
| 12           | 9+Mcherry         | 10            | 0               | 10                     | TBD                  | TBD                                           |
| <b>Total</b> | <b>218 Clones</b> | <b>120</b>    | <b>105</b>      | <b>15</b>              | <b>58</b>            | <b>8</b>                                      |

Table 2. Summary of *in vivo* screen.



**Figure 3. Representative mouse BC analysis.** (Top) Representative images of primary tumor and lungs from mice injected with cells stably expressing negative control (mCherry) or ORFs within pool 3. Large metastatic nodules noted by arrows. (Bottom). Percent BC enrichment detected from gDNA isolated from pooled injected cells (input), primary tumor cores (SubQ n=2) and macro-dissected lung metastases (n=2). Red boxes indicate putative metastasis drivers enriched only metastatic nodules. The blue box indicates a putative tumor growth driver enriched only in primary tumor.



Currently we are waiting to sacrifice mice injected with pool 12 (Table 2) and sequencing of the remaining BC-enriched samples is underway and expected to be completed by the time of our Final Report. **The Scott laboratory will continue BC enrichment analysis and present those findings, along with discussion on top identified metastasis drivers, in their Annual Report.**

### **Subaim 3.1. Clinical validation and prioritization of metastasis genes (proposed completion during Year 2).**

In this Aim we proposed to take forward identified drivers from the *in vitro* and *in vivo* screens for clinical validation and prioritization by analysis of their expression in publically available datasets (e.g., TCGA and others, as well as in-house datasets with clinical annotation). We also propose to begin functional and mechanistic studies of clinically validated high priority candidates.

Invasion drivers: Among the top scoring gene candidates reported in our first Annual Report were *YWHAZ*, *MBIP* and *SRP54*, which were all subsequently validated by performing invasion and migration assays in the standard 24-well Boyden chamber system. Consistent with the primary screen in 96-well format, we also reported significant increases in both migration and invasion for the 393P cells expressing each of the three genes compared to the mCherry negative control cells. These genes also score robustly in the 3D culture assays, providing additional evidence for their pro-invasion activity. **The Gibbons laboratory has continued secondary validation efforts on top hits from the invasion screen and will present those findings, along with results from the 3D culture assays, in their Annual and Final Reports.**

Metastasis drivers: We have also started validating identified metastasis drivers for their ability to induce lung metastasis by injecting them individually into syngeneic mice. These validation efforts will continue beyond the funding period of this project since BC analysis is still underway for the majority of pools (expected completion prior to Final Report). **The Gibbons laboratory will present secondary validation data on top hits from the *in vivo* screening effort in their Final Report.**

Survival analysis was performed on the 39 genes identified in the primary *in vitro* invasion assay by our collaborator Chad Creighton at Baylor College of Medicine. Based on this analysis, we filtered the candidate list of 39 genes for significant correlation with poor patient survival to give a final list of 10 genes (Table 3). These genes were then ranked according to percent over-amplification that also has significance associated with copy number versus transcript. Two of the candidates previously identified as significant drivers when mutated also scored in our primary invasion assay when the wild type gene is amplified. These 10 pro-invasion genes represent out top pro-invasion candidates. We will perform this analysis

| Rank | Gene ID | Chr     | Survival correlation<br>(p-value;1492 tumors) | TCGA % copy gain<br>(3 + copies) |
|------|---------|---------|-----------------------------------------------|----------------------------------|
| 1    | MYC     | 8q24.21 | 0.02                                          | Yes                              |
| 2    | YWHAZ   | 8q22.3  | 0.01                                          | Yes                              |
| 3    | KDELRL2 | 7p22.1  | 0.01                                          | Yes                              |
| 4    | DFNA5   | 7p15.3  | 0.00                                          | Yes                              |
| 5    | IMPAD1  | 8q12.1  | 0.02                                          | Yes                              |
| 6    | SNAI2   | 8q11.21 | 0.02                                          | Yes                              |
| 7    | DTL     | 1q32.3  | 0.00                                          | Yes                              |
| 8    | CCNE1   | 19q12   | 0.00                                          | Yes                              |
| 9    | CBLB*   | 3q13.11 | 0.03                                          | no                               |
| 10   | MAPK6*  | 15q21.2 | 0.00                                          | no                               |

**Table 3** . Top 10 genes which were identified to be highly invasive *in vitro*, shows high correlation with poor survival and demonstrates high frequency of amplification in the TCGA clinical samples.

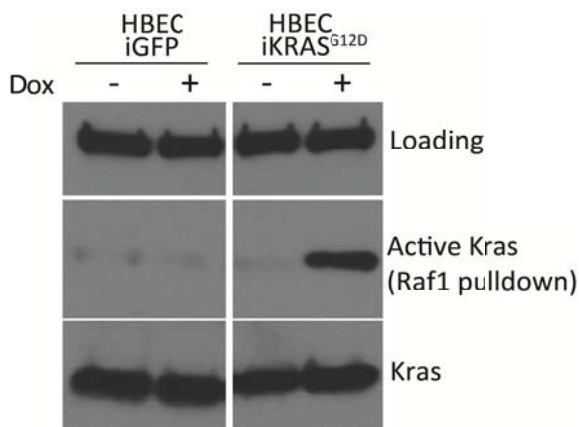
on ORFs enriched from our *in vivo* screens upon completion of BC sequencing. **We are also finalizing other *in silico* analyses that employ public and institutional (MD Anderson) datasets as described in our Statement of Work. These findings will be summarized in our Final Report.**

### **Subaim 3.2. Functional and mechanistic study of the lead metastasis genes (proposed completion during Year 2).**



For oncogenic and metastasis driver validation assays, we proposed to use cancer cell lines for appropriate expression or knock-down studies. Depending upon the particular genes identified in the screens and the observed phenotypes, we will use human NSCLC cancer cell lines or other cancer cell types as appropriate. In our first Annual Report we described our newly-developed immortalized human bronchial epithelial cell (HBEC) line engineered with signature aberrations (Cdk4, p53KD) and a doxycycline inducible *KRAS*<sup>G12D</sup> allele (i*KRAS*<sup>G12D</sup>; Fig.4). This lineage-specific model provides an important means to test the phenotypic role of ORFs identified from our *in vitro* and *in vivo* screens, with the specific ability to determine the dependence of those genes on oncogenic *KRAS*.

Completion of this Subaim is delayed due to the strategic delay in the *in vitro* screen (described above) and pending completion of the BC enrichment analysis for the *in vivo* screens. Nevertheless, we have already engineered individual HBEC i*KRAS*<sup>G12D</sup> cells that express 3 candidates: *YWHAZ*, *SNAI2*, *MBIP*. These cells are currently being validated for use in studies aimed at investigating potential regulatory pathways that these driver genes might be regulating to induce metastasis. These studies will enable us to identify potential targets for therapeutic intervention of metastatic lung cancers.



**Figure. 4. Immunoblot validation of HBEC-Kras model.** Immunoblot analysis of whole cell lysates extracted from HBEC cells engineered to express low levels of GFP or *KRAS*<sup>G12D</sup> afforded by used of the doxycycline (Dox) inducible system. Assessment Ras activation performed by RAF pull down assay.

## KEY RESEARCH ACCOMPLISHMENTS

- **ORF collection:** We expanded our screen platform to ~40,000 open reading frame (ORF; i.e., genes) clones. All ORFs are present in “entry” vectors compatible with Gateway-mediated recombineering, which allows high-throughput, robotics-driven ORF transfer to “destination” vectors for their expression in mammalian cells. Sequencing of all ORFs has enabled complete gene annotation, and our quality control criteria mandates 100% amino acid concordance with NCBI Reference entries.
- **High-throughput mutagenesis and molecular barcoding (HiTMMoB):** Enables high-throughput mutagenesis and molecular barcoding of our extensive ORF collection.
- **Development of a high-throughput *in vitro* invasion screen with dynamic range:** Facilitates rapid and quantifiable cellular phenotyping of the genes in the screen.
- **Robust 3D invasion assay:** Better mimics the 3D nature and extracellular matrix components found *in vivo*, while allowing manipulation of the matrix and easy scoring of the cellular phenotype.
- **Positive *in vivo* growth and metastasis screen:** Ability to directly test the *in vivo* role of genes on primary tumor growth and metastasis in a medium-throughput, pooled fashion due to

the innovative barcoding techniques, use of sensitive sequencing and the positive selection of the *in vivo* screen. Multiple hits identified for functional follow-up experiments and entry into target validations assays designed to identify new drug targets.

- **Inducible *KRAS*<sup>G12D</sup> HBEC cell model:** Provides proper genetic context in which to functionally examine *KRAS* effectors.

## **REPORTABLE OUTCOMES**

- **pLentiEF6.3-puro:** We created a specialized lentiviral destination vector for the proposed studies. This vector permits accepts human wild-type and mutant ORFs via high-throughput recombination in the presence of DNA barcodes. The vector has an EF1a promoter compatible with expression in mouse and human cells, and also contains a puromycin selection cassette necessary for the cell lines used in this study.
- **Pro-invasion genes:** Primary screens and secondary validation have revealed 39 genes that robustly drive cell invasion.
- **Pro-metastasis genes:** Primary screening has already identified 8 novel metastasis genes not previously linked to lung cancer progression. A full list of scoring genes among the candidates screened will be provided in our Final Report and resulting publications.
- **Mutant clone repository:** Cataloging of all sequenced-verified aberration clones constructed by this project which will be deposited at the DNASU Plasmid Repository (Arizona State University) to facilitate their distribution to the community thus maximizing their use.
- **Generation of an isogenic HBEC cell line with inducible mutant *KRAS* expression:** This cell system will allow us to test the oncogenic potential of the positive genes from the screen and the importance of the mutant *KRAS* background for their function.

## **CONCLUSIONS**

Our overall goal with this project is to establish a pipeline of robust screening techniques to functionally prioritize the data emerging from large-scale genomics efforts in lung cancer. Using a combination of *in vitro* and *in vivo* screens we will be able to identify and validate oncogene and metastasis drivers, explore the mechanistic basis for their function, and generate the pre-clinical cell and animal models needed for therapeutic targeting studies. As outlined in the Body of this report, we have successfully constructed the necessary libraries for this work, completed the *in vitro* invasion screens, nearing completion of *in vivo* screening BC analysis and have initiated mechanistic studies of the lead candidate genes using our HBEC-i*KRAS*<sup>G12D</sup> cell model. We have made great technical inroads and view this project as a robust start to further screening of the TCGA data (which has only recently been released) that will extend past the life of this particular grant. We also feel that these techniques can be broadly implemented for functionalization of genomic data for other tumor types.

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## **APPENDICES**

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